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GMP synthetase from plants

The pres nt invention relates to the identification of plant GMP synthetase (guanosine-monophosphate synthetase) as novel target for herbicidal agents. The present invention further relates to DNA sequences coding for a polypeptide having GMP synthetase (EC 6.3.5.2) activity. The invention moreover relates to the use of a nucleic acid coding for a protein having GMP synthetase activity of plant origin for producing an assay system for identifying inhibitors of GMP synthetase having a herbicidal action, and to inhibitors of plant GMP synthetase identified using this assay system. The invention further relates to the use of the nucleic acid coding for plant GMP synthetase for producing plants with increased resistance to inhibitors of GMP synthetase, and for producing plants with a modified content of guanosine nucleotides. The invention additionally relates to a method for eliminating unwanted plant growth, which comprises treating the plants to be eliminated with a compound which specifically binds to GMP synthetase encoded by a DNA sequence SEQ-ID No. 1 or a DNA sequence hybridizing with the latter, and inhibits the function thereof.

Plants are able to synthesize their cellular components from carbon dioxide, water and inorganic salts.

This process is possible only through the use of biochemical reactions to synthesize organic substances. It is necessary for plants to synthesize de novo the nucleotides as constituents of nucleic acids.

Especially in rapidly growing plant tissues it is necessary for nucleotides as constituents of the nucleic acids DNA and RNA to be synthesized by multistage metabolic pathways. Nucleotides are moreover linked in with virtually all metabolic pathways. Nucleoside triphosphates, especially ATP, drive many energy-expending reactions in cells. Adenine nucleotide additionally occurs as component in essential coenzymes such as coenzyme A and nicotinamide and flavin coenzymes, which are involved in many cellular conversions. Guanosine nucleotides give a reaction direction to various cellular processes such as protein translation, microtubule assembly, vesicular transport, signal transduction and cell division. In addition, nucleotides are the starting metabolites for the biosynthesis of methylxanthines such as caffeine and theobromine, especially in the Rubiaceae and Theaceae families of plants.

Purine nucleotides are formed in microorganisms, animals and plants de novo in the same way starting from phosphoribosyl pyrophosphate (PRPP). IMP is synthesized in a 10-stage reaction sequence. IMP can be converted in subsequent reactions by adenylosuccinate synthetase and adenylosuccinate lyase into AMP. In the synthesis of GMP there is initial conversion of IMP by IMP dehydrogenase into XMP which is aminated by GMP synthetase to give GMP, see Fig. 1.

Genes which code for GMP sythetase [sic] have been isolated from various organisms.

The compartmentation of the purine biosynthetic pathway in plants has not to date been extensively investigated. The nitrogen which is fixed in the form of glutamine and aspartate in the root nodules of legumes is firstly converted via the de novo synthetic pathway into purines. This pathway is localized in the plastids in the root nodules of *Glycine max* and *Vigna unguiculata* L. (Boland and Schubert, Arch. Biochem. Biophys. 220 (1983), 179-187; Shelp et al., Arch. Biochem. Biophys. 224 (1983), 429-441). However, more recent investigations have shown that enzyme activitites of the purine biosynthetic pathway are also to be found in mitochondria in the root nodules of *Vigna ungiculata* [sic] (Atkins et al., Plant Physiology 113 (1997), 127-135; Smith et al., Plant Molecular Biology 36 (1998), 811-820).

The regulation of this synthetic pathway has to date been investigated only in microorganisms and animals and comprises transcription control, end-product inhibition and allosteric regulation. The enzyme PRPP amidotransferase (PRPP ATAse) of the second reaction step is attributed with a key position in the animal as well as the plant system and is subject to allosteric regulation by the end products IMP, AMP and GMP (Reynolds et al., Archives of Biochemistry and Biophysics 229 (1984), 623-631).

GMP-Synthetase also plays a part in relation to the balanced synthesis of guanosine nucleotides and adenosine nucleotides because ATP is a substrate of GMP synthetase.

Since plants are dependent on a functioning nucleotide metabolism, this metabolism is obviously a possible target of novel herbicides. In fact, agents with an inhibitory ffect on enzymes of de novo purine biosynthesis hav alr ady b n described. An example which may be mention d is 5'-phosphohydantocidin which inhibits an enzyme of plant purine metabolism, adenylosuccinate synthetase (ASS) (Siehl et al., Plant Physiol. 110 (1996), 753-758). Inhibitors for enzymes of

this metabolic pathway also xist from animals and microorganisms. Folate analogs inhibit various folate-dependent reactions, inter alia the enzyme GAR transformylase and hav antiproliferative, antiinflammatory and immunosuppressant effects. Mycophenolate (MPA), as an inhibitor of IMP dehydrogenase, has antimicrobial, antiviral and immunosuppressant effects. (Kitchin et al., Journal of the American Academy of Dermatology 37 (1997), 445-449).

Demonstration of the suitability of an enzyme as herbicide target can be shown, for example, by reducing the enzyme activity by means of the antisense technique in transgenic plants. If reduced growth is brought about in this way, it can be concluded that the enzyme whose activity has been reduced is a suitable site of action of herbicidal agents. This has been shown by way of example for acetolactate synthase on transgenic potato plants (Höfgen et al., Plant Physiology 107 (1995), 469-477).

It is an object of the present invention to prove that GMP synthetase in plants is a suitable herbicidal target, to isolate a complete plant cDNA coding for the enzyme GMP synthetase and functional expression thereof in bacterial or eukaryotic cells, and to produce an efficient and simple GMP synthetase assay system for carrying out the inhibitor-enzyme binding studies.

We have found that this object is achieved by isolation of a gene coding for the plant enzyme GMP synthetase, the production of antisense constructs of GMP synthetase, and functional expression of the GMP synthetase in bacterial or eukaryotic cells.

One aspect of the present invention relates to the isolation of a full-length cDNA coding for a functional glutamine-hydrolyzing GMP synthetase (EC 6.3.5.2.) from tobacco (*Nicotiana tabacum*).

A first aspect of the present invention is a DNA sequence SEQ-ID NO:1 comprising the coding region of a plant GMP synthetase from tobacco, see Example 1.

Another aspect of the invention is a DNA sequence SEQ-ID No. 3 comprising a portion of the coding region of a plant GMP synthetase from Physcomitrella patens, see Example 2.

Further aspects of the invention are DNA sequences which are derived from SEQ-ID NO: 1 or SEQ-ID No: 3 or hybridize with one of thes s quences and code for a protein which has the biological activity of a GMP synthetase.

Tobacco plants of the lin Nicotiana tabacum cv. Samsun NN harboring an antisense construct of GMP synthetase have been characterized in detail. The plants show growth retardation to differing extents. The transgenic lines, and the progeny as 1st and 2nd generation showed reduced growth in soil. In plants with reduced growth it was possible to detect a reduced, compared with the wild-type, GMP-7M RNA amount in the Northern hybridization. It was also possible in a Western blot experiment to detect a reduced amount, compared with wild-type plants, of GMP synthetase in the transgenic lines, see Example 7. A correlation can be found in the growth retardation and reduction in the amount of GMP synthetase protein. This clear association demonstrates for the first time that GMP is unambiguously a suitable target protein for herbicidal agents.

In order to be able to find efficient inhibitors of plant GMP synthetase, it is necessary to provide suitable assay systems wih which inhibitor-enzyme binding studies can be carried out. For this purpose, for example, the complete cDNA sequence of the GMP synthetase from tobacco is cloned in an expression vector (pQE, Qiagen) and is expressed in E. coli, see Example 4.

An alternative possibility is, however, to express the expression cassette comprising a DNA sequence SEQ-ID No. 1 for example in other bacteria, in yeasts, fungi, algae, plant cells, insect cells or mammalian cells, see Example 5.

The GMP synthetase protein expressed with the aid of the expression cassette according to the invention is particularly suitable for finding inhibitors specific for GMP synthetase.

For this purpose, the plant GMP synthetase can be employed, for example, in an enzyme assay in which the activity of the GMP synthetase is measured in the presence and absence of the agent to be tested. Qualitative and quantitative information about the inhibitory characteristics of the agent to be tested is obtainable from comparison of the two activity determinations, see Example 8.

The assay system according to the invention can be used for rapid and simple testing of a large number of chemical compounds for herbicidal properties. The method allows reproducibl s lection from a large number of substances specifically of those having a potent effect in order then to carry out other, more intensive tests which are familiar to the skilled worker on these substanc s.

A further aspect of th invention is a method for identifying substances having a herbicidal action, which inhibit the GMP synthetase activity in plants, consisting of

a) preparation of transgenic plants, plant tissues, or plant cells which comprise an additional DNA sequence coding for an enzyme having GMP synthetase activity and are able to overexpress an enzymatically active GMP synthetase;

b) application of a substance to transgenic plants, plant cells, plant tissues or plant parts and to untransformed plants, plant cells, plant tissues or plant parts;

c) determination of the growth or survivability of the transgenic and untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance; and

d) comparison of the growth or survivability of the transgenic and untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance;

where suppression of the growth or survivability of the untransformed plants, plant cells, plant tissues or plant parts without, however, greatly suppressing the growth or the survivability of the transgenic plants, plant cells, plant tissues or plant parts demonstrates that the substance from b) shows herbicidal activity and inhibits the GMP synthetase enzymic activity in plants.

A further aspect of the invention is a method for identifying inhibitors of plant GMP synthetases, with potential herbidical action, by cloning the gene of a plant GMP synthetase, bringing about overexpression in a suitable expression cassette - for example in insect cells, opening the cells and employing the cell extract directly or after concentration or isolation of the enzyme GMP synthetases in an assay system for measuring the enzymic activity in the presence of low molecular weight chemical compounds.

A further aspect of the invention comprises compounds having a herbicidal action which can be identified using the assay system described above.

A further aspect of the invention is a method for eliminating unwanted plant growth, which comprises treating the plants to be eliminated with a compound which specifically binds to plant GMP

synthetase and inhibits the function thereof.

Inhibitors of GMP synthetase with a herbicidal action can be used as defoliants, desiccants, haulm destroyers and, in particular, weedkillers. Weeds mean in the widest sense all plants which grow where they are unwanted. Whether the agents found with the aid of the assay system according to the invention act as total or selective herbicides depends inter alia on the application rate.

Inhibitors of GMP synthetase with a herbicidal action can be used, for example, to control the following weeds:

Dicotyledonous weeds of the genera:
Sinapis, Lepidium, Galium, Stellaria, Matricaria, Anthemis, Galinsoga, Chenopodium, Urtica, Senecio, Amaranthus, Portulaca, Xanthium, Convolvulus, Ipomoea, Polygonum, Sesbania, Ambrosia, Cirsium, Carduus, Sonchus, Solanum, Rorippa, Rotala, Lindernia, Lamium, Veronica, Abutilon, Emex, Datura, Viola, Galeopsis, Papaver, Centaurea, Trifolium, Ranunculus, Taraxacum.

Monocotyledonous weeds of the genera:
Echinochloa, Setaria, Panicum, Digitaria, Phleum, Poa, Festuca, Eleusine, Brachiaria, Lolium, Bromus, Avena, Cyperus, Sorghum, Agropyron, Cynodon, Monochoria, Fimbristylis, Sagittaria, Eleocharis, Scirpus, Paspalum, Ischaemum, Sphenoclea, Dactyloctenium, Agrostis, Alopecurus, Apera.

Another aspect of the invention comprises expression cassettes whose sequence codes for a GMP synthetase from tobacco or functional equivalent thereof. The nucleic acid sequence may in this connection be, for example, a DNA or a cDNA sequence.

Another aspect of the invention is an expression cassette comprising a DNA sequence SEQ-ID No. 3 coding for a portion of the plant GMP synthetase from Physcomitrella patens.

The expression cassettes according to the invention additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette according to the invention comprises a promoter upstream, i.e. at the 5' end of the coding sequence, and a polyadenylation signal downstream, i.e. at the 3' end, and, where appropriate, further regulatory elements which are operatively linked to the GMP synthetase gene coding sequence lying between them. An operative linkage means the sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in

such a way that each of the regulatory elements is able to perform its function as intended on expression of the coding sequence.

An expression cassette according to the invention is produced by fusing a suitable promoter with a suitable GMP synthetase DNA sequence and a polyadenylation signal by conventional recombination and cloning techniques as described, for example, in J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

Another aspect of the invention comprises functionally equivalent DNA sequences which code for a GMP synthetase and which have, based on the complete length of the DNA sequence, a sequence homology with the DNA sequence SEQ-ID NO: 1 or SEQ-ID No. 3 of from 40 to 100%.

A preferred aspect of the invention comprises functionally equivalent DNA sequences which code for a GMP synthetase and which have, based on the complete length of the DNA sequence, a sequence homology with the DNA sequence SEQ-ID NO: 1 or SEQ-ID No. 3 of from 60 to 100%.

A particularly preferred aspect of the invention comprises functionally equivalent DNA sequences which code for a GMP synthetase and which have, based on the complete length of the DNA sequence, a sequence homology with the DNA sequence SEQ-ID NO: 1 or SEQ-ID No. 3 of from 80 to 100%.

Functionally equivalent sequences which code for a GMP synthetase are, according to the invention, sequences which, despite a different nucelotide sequence, still have the desired functions. Functional equivalents thus comprise naturally occurring variants of the sequences described herein, and artificial nucleotide sequences, for example obtained by chemical synthesis, which are adapted to the codon usage of a plant.

A functional equivalent also means in particular natural or artificial mutations of an originally isolated sequ nc which codes for a GMP synthetase and additionally shows the required function. Mutations comprise substitutions, additions, deletions, transpositions or insertions of one or more nucleotide residues.

Thus, for exampl , the pres nt invention also includes nucleotide sequences obtained by modification of the nucleotide sequence. The aim of such a modification may be, for example, further localization of the coding sequence present therein or, for example, insertion of further restriction enzyme cleavage sites.

Functional equivalents are also variants whose function has been attenuated or enhanced by comparison with initial gene or gene fragment.

The expression cassette according to the invention can also be employed for transforming bacteria, cyanobacteria, yeasts, filamentous fungi and algae with the aim of producing adequate amounts of the enyzme GMP synthetase.

A further aspect of the invention is a protein from tobacco which has the amino acid sequence SEQ-ID NO: 2 or derivatives or portions of this protein with GMP synthetase activity.

Another aspect of the invention comprises plant proteins with GMP synthetase activity having an amino acid sequence homology with the tobacco GMP synthetase of 20 - 100% identity.

Preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the tobacco GMP synthetase of 50 - 100% identity.

Particularly preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the tobacco GMP synthetase of 80 - 100% identity.

Another aspect of the invention comprises plant proteins with GMP synthetase activity having an amino acid sequence homology with the Physcomitrella patens GMP synthetase of 20 - 100% identity.

Preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the Physcomitrella patens GMP synthetase of 50 - 100% identity.

Particularly preferred plant proteins with GMP synthetase activity have an amino acid sequence homology with the Physcomitrella patens GMP synthesase of 80 - 100% identity.

A further object of the invention was overexpr ssion of the GMP synthetase gene in plants to produce plants which are tolerant of inhibitors of GMP synthetase.

Overexpression of the gene sequence SEQ-ID NO: 1 coding for a GMP synthetase in a plant achieves increased resistance to inhibitors of GMP synthetase. The transgenic plants produced in this way are likewise an aspect of the invention.

The efficiency of expression of the transgenically expressed GMP synthetase gene can be measured, for example, in vitro by shoot meristem propagation or by a germination test. In addition, a change in nature and level of the expression of the GMP synthetase gene and the effect thereof on the resistance to inhibitors of GMP synthetase can be tested on test plants in glasshouse experiments.

An additional aspect of the invention comprises transgenic plants transformed with an expression cassette according to the invention comprising DNA sequence SEQ-ID No. 1, which has become tolerant of inhibitors of GMP synthetase due to additional expression of DNA sequence SEQ-ID No. 1, and to transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, soybean, rice, cotton, sugar beet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species, and legumes.

An alteration in the nucleotide content in plants may be beneficial in various cases. For example, nucleotides are added to plant-based babyfood products in order to achieve a nutrient solution corresponding to breast milk. In addition, an optimized nucleotide content would be sensible in the enteral feeding of patients. A reduced purine nucleotide content in plants of foods relevance is relevant to the dietary feeding of patients with gout. Nucleotides also have flavor-forming and flavor-enhancing effects so that an altered nucleotide content has effects on the taste properties of plants.

A further aspect of the invention therefore comprises plants which have a modified content of guanosine nucleotides after expression of the DNA sequence SEQ-ID NO: 1 or SEQ-ID No: 3 in the plant.

A plant with a modified content of guanosine nucleotides is based, for example, by expression of an additional DNA sequence SEQ-ID No. 1 or 3 in the sense or antisense orientation in the plant. A modified content of guanosine nucleotides means that it is possible to produce plants with an increased content of guanosine nucleotides in the case of the sense orientation and

plants with a reduced content of guanosine nucleotid s in the case of the sense orientation (cosuppression) or antisens orientation.

Increasing the content of guanosine nucleotides means, for example, within the scope of the present invention the artificially applied capability of increased biosynthesis of guanosine nucleotides owing to functional overexpression of the GMP synthetase gene in the plant compared with the plant which has not been genetically manipulated for the duration of at least one plant generation.

A further aspecet of the invention is the use of plant GMP synthetases to alter the concentrations of methylxanthines in plants.

Particularly preferred sequences are those which ensure targeting in the apoplasts, in plastids, the vacuoles, the mitochondrion, the endoplasmic reticulum (ER) or, through the absence of appropriate operative sequences, ensure retention in the compartment of production, the cytosol (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR, see Example 6.

Suitable in principle as promoter for the expression cassette according to the invention is every promoter able to control expression of foreign genes in plants. It is particularly preferred to use a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from Blumenkohl mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particuliarly preferred. This promoter contains various recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the introduced gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette according to the invention may also comprise a chemically inducible promoter through which it is possible to control expression of the exogenous GMP synthetase gene in the plant at a particular point in time. Promoters of this type, such as, for example, PRP1 promoter (Ward et al., Plant. Mol. Biol. (1993) 22, 361-366), a promoter inducible b y salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP 388186), a tetracycline-inducible (Gatz et al., Plant J. (1992) 2, 397-404), an abscisic acid-inducible (EP0335528) or an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter are

described in the literature and can, inter alia, be used.

Further particularly preferred promot rs are those which ensure expression in tissues or plant parts in which the biosynthesis of purines or their precursors takes place. Particular mention may be made of promoters which ensure leaf-specific expression. Mention should also be made of the promoter of the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J., (1989) 8, 2445-2451).

It is possible with the aid of a seed-specific promoter to express a foreign protein stably up to a content of 0.67% of the total soluble seed protein in the seed of transgenic tobacco plants (Fiedler and Conrad, Bio/Technology (1995) 10, 1090-1094). The expression cassette according to the invention can therefore comprise, for example, a seed-specific promoter (preferably the phaseolin promoter, the USP or LEB4 promoter), the LEB4 signal peptide, the gene to be expressed and an ER retention signal.

The inserted nucleotide sequence coding for a GMP synthetase can be prepared synthetically or be obtained naturally or comprise a mixture of synthetic and natural DNA components. In general, synthetic nucleotide sequences are produced with codons preferred by plants. These codons preferred by plants can identified by codons which have the highest protein frequency and are expressed in most plant species of interest. To prepare an expression cassette it is possible to manipulate various DNA fragments in order to obtain a nucleotide sequence which expediently reads in the correct direction and is equipped with a correct reading frame. Adaptors or linkers can be attached to the framework to connect the DNA fragments together.

Artificial DNA sequences are also suitable as long as they confer, as described above, the required property of increasing the content of guanosine nucleotides in the plant through overexpression of the GMP synthetase gene in crop plants. Such artificial DNA sequences can be found, for example, by translation back from proteins having GMP synthetase activity and constructed by molecular modeling, or by *in vitro* selection. Particularly suitable coding DNA sequences are those obtained by translation back from a polypeptide sequence in accordance with the codon usage specific for the host plant. The specific codon usage can easily be found by a skilled worker familiar with methods of plant genetics by computer analyses of oth r known genes of the plant to be transformed.

Further suitable equivalent nucleic acid sequences according to the invention which may be mentioned are sequences coding for fusion prot ins where one constituent of the fusion protein is a plant GMP synthetase polypeptide or a functionally equivalent portion thereof. The second part of the fusion protein can be, for example, another polypeptide with enzymatic activity or an antigenic polypeptide sequence with whose aid it is possible to detect GMP synthetase expression (e.g. myc tag or his tag). However, this is preferably a regulatory protein sequence such as, for example, a signal or transit peptide which guides the GMP synthetase protein to the desired site of action.

The promoter regions according to the invention and the terminator regions ought expediently to be provided in the direction of transcription of a linker or polylinker containing one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, usually 1 to 8, preferably 2 to 6, restriction sites. The size of the linker within the regulatory region is generally less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter according to the invention may be both native or homologous and foreign or heterologous to the host plant. The expression cassette according to the invention comprises in the 5'-3' direction of transcription the promoter according to the invention, any suitable sequence and a region for the transcriptional termination. Different termination regions can be exchanged for one another if desired.

It is furthermore possible to employ manipulations which provide suitable restriction cleavage sites or delete the excess DNA or restriction cleavage sites. Where the insertions, deletions or substitutions such as, for example, transitions and transversions are considered, it is possible to use *in vitro* mutagenesis, primer repair, restriction or ligation. In the case of suitable manipulations such as, for example, restriction, chewing-back or filling in of overhangs for blunt ends, it is possible to make complementary ends of the fragments available for the ligation.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, in particular of gene 3 of the T-DNA (octopine synthase) of Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 ff) or functional equivalents.

To transform a host plant with a DNA coding for a GMP synthetase, an expression cassette according to the invention is incorporated

as insert into a recombinant vector whose vector DNA contains additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described inter alia in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chapters 6/7, pages 71-119.

The transfer of foreign genes into the genome of a plant is referred to as transformation. The methods used for this purpose are those described for the transformation and regeneration of plants from plant tissues or plant cells for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic approach with the gene gun, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and gene transfer mediated by agrobacterium. The methods mentioned are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991) 205-225). The construct to be expressed is preferably cloned into a vector which is suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711).

Agrobacteria transformed with an expression cassette according to the invention can likewise be used in known manner for transforming plants, especially plants such as cereals, corn, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and various tree, nut and vine species, and legumes, for example by bathing wounded leaves or pieces of leaf in a solution of agrobacteria and then cultivating in suitable media.

The site of pyrimidine biosythesis is generally the leaf tissue, so that leaf-specific expression of the GMP synthetase gene is sensible. However, it is obvious that pyrimidine biosynthesis need not be confined to leaf tissue but may also take place in all other parts of the plant, for example in fat-containing seeds, tissue-specifically.

In addition, constitutive expression of the exogenous GMP synthetase gene is advantageous. However, on the other hand, inducible expression may also appear desirable.

Using the recombinant and cloning techniques quoted above, the xpression cassettes according to th invention can be cloned into suitable vectors which make it possible to replicate them,

for example into E. coli. Suitable cloning vectors are, inter alia, pBR322, pUC series, M13mp series and pACYC184. Binary vectors abl to replicate both in E. coli and in agrobact ria ar particularly suitable.

A further aspect of the invention relates to the use of an expression cassette according to the invention for transforming plants, plant cells, plant tissues or parts of plants. The aim of the use is preferably to increase the GMP synthetase content in the plant.

This may involve, depending on the chosen promoter, expression specifically in the leaves, in the seeds or other parts of the plant. Such transgenic plants, their propagation material and their plant cells, tissues or parts form a further aspect of the present invention.

The invention is illustrated by the Examples which now follow, but is not confined to these:

Examples

Methods of genetic manipulation on which the examples are based:

General cloning methods

Cloning methods such as, for example, restriction cleavage, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of Escherichia coli cells, cultivation of bacteria and sequence analysis of recombinant DNA were carried out as described by Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6).

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using a laser fluorescence DNA sequencer supplied by ABI by the method of Sanger (Sanger et al. (1977), Proc. Natl. Acad. Sci. USA74, 5463-5467). Fragments resulting from a polymerase chain reaction were sequenced and checked to avoid polymerase errors in constructs to be expressed.

The chemicals used wer purchased, unless oth rwis mentioned, in analytical quality from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deis nhofen). Solutions were made up using prepared, pyrogen-free wat r,

referred to as H_2O in the subsequent text, from a Milli-Q wat r treatment system (Millipore, Eschborn). R striction endonucleases, DNA-modifying enzymes and molecular biology kits were purchased from AGS (Heidelberg), Amersham (Braunschweig), Biometra (Göttingen), Roche (Mannheim), Genomed (Bad Oeynhausen), New England Biolabs (Schwalbach/Taunus), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt), Pharmacia (Freiburg), Qiagen (Hilden) and Stratagene (Heidelberg). They were used in accordance with the manufacturers' instruction unless mentioned otherwise.

The strains of bacteria used hereinafter (E. coli, XL-1 Blue) were purchased from Stratagene. E. coli AT 2465 was purchased from the coli genetic stock center (Yale University, New Haven). The agrobacterial strain used for the plant transformation (Agrobacterium tumefaciens, C58C1 with the plasmid pGV2260 or pGV3850kan) has been described by Deblaere et al. (Nucl. Acids Res. 13 (1985) 4777). An alternative possibility is also to employ the agrobacterial strain LBA4404 (Clontech) or other suitable strains. Vectors which can be used for the cloning are pUC19 (Yanish-Perron, Gene 33(1985), 103-119) pBluescript SK- (Stratagene), pGEM-T (Promega), pZerO (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12(1984) 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230).

Example 1

Isolation of a cDNA of the guaA gene, coding for a GMP synthetase from tobacco.

An expressed sequence tag (EST) from Arabidopsis thaliana (EST F14426) which, on a partial reading frame, encodes a polypeptide of 68 amino acids with 60% similarity with a GMP synthetase from *Helicobacter pylori* was subjected to partial 5'-terminal sequencing. The oligonucleotides 5'-aag gat cca agc tct aag acc cta tcc-3' and 5'-tta gat ctt tat tcc cat tcg atg g-3' from the 5'- and 3'-terminal sequences were used for amplification by a polymerase chain reaction (PCR) of a 1000 bp cDNA fragment with EST F14426 as template in a Perkin Elmer DNA thermal cycler. The reaction mixture contained 0.1 ng/µl cDNA from tobacco, 0.5 µM of the appropriate oligonucleotides, 200 µM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM $MgCl_2$) and 0.02 U/µl Taq polymerase (Perkin Elmer).

The amplification conditions wer set as follows:

Annealing temperature:	52°C, 1 min

Denaturation temperature:	92°C, 1 min
Elongation temperature:	72°C, 1.5 min
Number of cycles:	30

The fragment was employed for screening a cDNA library from callus tissue of *Nicotiana tabacum* (variety Samsun NN) in the vector ZAP Express. For this purpose, 2.5 x 10^5 lambda phages from the cDNA library were plated out on agar plates with E. coli XL1-Blue as bacterial strain. The phage DNA was transferred by standard methods (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0=87969-309-6) to nitrocellulose filters (Gelman Sciences) and fixed on the filters. The hybridization probe used was the PCR fragment described above which had been radiolabeled using a multiprime DNA labeling system (Amersham Buchler) in the presence of α-^{32}P-dCTP (specific activity 3000 Ci/mmol) in accordance with the manufacturer's information. The hybridization of the membranes took place after prehybridization at 60°C in 3 x SSPE, 0.1% sodium dodecyl sulfate (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.02% Ficoll 400 (w/v) and 50 mg/ml calf thymus DNA for 12-16 hours (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6). The filters were then washed in 2 x SSPE, 0.1% sodium dodecyl sulfate (w/v) at 60°C for 60 minutes. Positively hybridizing phages were visualized by autoradiography and purified and isolated by standard techniques.

It was possible to identify and purify 13 hybridizing signals. After restriction analysis, the clones GMP-6 and GMP-7M were selected for double-stranded sequencing. Evaluation of the sequencing data showed that the clone GMP-7M with a length of 1973 bp contained a complete reading frame of 1614 bp which codes for a protein of 538 amino acids with a calculated molecular weight of 60.1 KDa (SEQ-ID No. 1). In front of the presumed start codon there is a stop codon in the same reading frame, which suggests that GMP-7M is a full-length cDNA. GMP-7M thus represents the first full-length plant cDNA of a GMP synthetase. GMP-6 is a partial clone which is 217 nucleotides shorter than GMP-7M on the 5'. GMP-7M shows similarities with GMP synthetases from microorganisms and animals. Apart from the partial amino acid sequence encoded on EST F14426 there are no other sequences from plants with homology with GMP synthetases in the databases. The greatest similarity (62%) is with a GMP synthetase from *Helicobacter pylori*. It is also evident that the similarities between the C termini of the GMP synthetases are greater than those in th region of the N termini. The N terminus of the GMP-7M amino acid sequence corresponds with the N termini of GMP synthetases from other organisms such as *E. coli* and

Synechocystis sp. (Table 1). GMP-7M has no marked signal sequences (found by the program PSORT, Nakai, K., Institute for Molecular and Cellular Biology, Osaka University, Japan), which might indicate a cytosolic localization of th protein.

Table 1

Sequence comparison of GMP synthetases from Nicotiana tabacum (guaA_N.t = GMP-7M), Arabidopsis thaliana (guaA_est_A.t, Genbank No. F14426), E.coli (guaA_e.c, Genbank No. 146276), Synechocystis sp. (guaA_syn, Genbank No. 1001583), Helicobacter pylori (guaA_h.p, Genbank No. 3122166), Homo sapiens (guaA_human, Genbank No. 1708072).

```
                1                                                   50
     guaA_N.t   ---------- ------MEPQ TQAKKSNLVL ILDYGSQYTH LITRRIRSLS
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
     guaA_e.c   ---------- ---------m tenihkhril ildfgsqytq lvarrvrelg
     guaA_syn   mttqipvppv vsdqalpdri sdrlkgqiiv ildfgsqyse liarrirete
     guaA_h.p   ---------- ---------- -------mil vldfgsqytq liarrlrerg
   guaA_human   -malcngdsk lenaggdlkd ghhhyegavv ildagaqygk vidrrvrelf
               51                                                  100
     guaA_N.t   IFSLTINGTS SLDSIKELDP RVIILSGGPH SVHADGAPCF PPGFIEYVES
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
     guaA_e.c   vycelwawdv teaqirdfnp sgiilsggpe stteenspra pq....yvfe
     guaA_syn   vysevlsyrt taqqlreikp kgiilsggpn svydqgapec dp....eifq
     guaA_h.p   iyteivpffe sieniqkkap kglilsggpa svyakdaykp sg....kifd
   guaA_human   vqseifplet pafaikeqgf raiiisggpn svyaedapwf dpa....ift
               101                                                 150
     guaA_N.t   RGIHVLGICY GLQLIVQKLG GVVKIGEKHE YGRMEIEVGK NVV....GGL
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
     guaA_e.c   agvpvfgvcy gmqtmamqlg ghveasnere fgyaqvevvn dsalvrgied
     guaA_syn   lgvpvlgvcy gmqlmvkqlg grverakrge ygkaslhidd ptdlltnven
     guaA_h.p   lnvpilgicy gmqylvdffg gvvvganeqe fgkavleitq nsvifegv..
   guaA_human   igkpvlgicy gmqmmnkvfg gtvhkksvre dgvfnisvdn tcslfrglqk
               151                                                 200
     guaA_N.t   FGNTEIGDKQ VVWMSHGDEA VKLPEGFEVV ARSSQGAVAA IENRERRFYG
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
     guaA_e.c   altadgkpll dvwmshgdkv taipsdfitv astescpfai maneekrfyg
     guaA_syn   dst..... .mwmshgdsc vdlptgfeil ahtdntpcaa iadhqkalfg
     guaA_h.p   ......kiks lvwmshmdkv ielpkgfttl akspnsphca iengk..ifg
   guaA_human   ........ee vvllthgdsv dkvadgfkvv arsgni.vag ianeskklyg
               201                                                 250
     guaA_N.t   LQYHPEVTHS TEGMRTLRHF LFDVCGVTAG WKMEDVLEEE IKVIKGMVGP
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
     guaA_e.c   vqfhpevtht rqgmrmlerf vrdicqceal wtpakiidda varireqvg.
     guaA_syn   vqfhpevvhs vggialirnf vyhichcept wttaafiees irevrsqvg.
     guaA_h.p   lqfhpevvqs eeggkilenf allvcgcekt wgmqhfaqre iarlkekia.
   guaA_human   aqfhpevglt engkvilknf lydiagcsgt ftvqnrelec ireikervgt
               251                                                 300
     guaA_N.t   EDHVICALSG GVDSTVAAKL VHKAIG.DRL HCVFVDNGLL RYKERERVME
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
     guaA_e.c   ddkvilglsg gvdssvtaml lhraig.knl tcvfvdngll rlneaeqvld
     guaA_syn   drrvllalsg gvdsstlafl lhraig.dnl tcmfidqgfm rkgep rlve
     guaA_h.p   nakvlcavsg gvdstvvatl lhraik.dnl iavfvdhgll rknekervqa
   guaA_human   s.kvlvllsg gvdstvctal lnralnqeqv iavhidngfm rkresqsvee
```

```
                301                                                  350
      guaA_N.t  LPEK..... .......... ...RLHLPVT CVDATEEFLS KLKGVTEPEM
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
      guaA_e.c  mfgd..... .......... ...hfglniv hvpa drfls alagendpea
      guaA_syn  lfdh..... .......... ...qfhipvq yvnardrflk qlegvtdpee
      guaA_h.p  mfkd..... .......... ....lkipln tidakevfls klkgvsepel
    guaA_human  alkklgiqvk vinaahsfyn gtttlpisde drtprkrisk tlnmttspee
                351                                                  400
      guaA_N.t  KRKIIGKEFI NIFDLFAHDV EEKVGKKPSY LVQGTLYPDV IESC...PPP
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
      guaA_e.c  krkiigrvfv evfd..eeal k...ledvkw laqgtiypdv iesaas....
      guaA_syn  krrlighefi qvfe..eesn r...lgpfdy laqgtlypdv iesadsnvdp
      guaA_h.p  krkiigetfi evfe..keak khhlkgkief laqgtlypdv iesvsv....
    guaA_human  krkiigdtfv ki..anevig emnlkpeevf laqgtlrpdl iesasl....
                401                                                  450
      guaA_N.t  GSGRTHSHTI KSHHNVGGLP KDMKL..KLI EPLKLLFKDE VRELGKILDI
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
      guaA_e.c  atgk..ahvi kshhnvgglp kemkm..glv eplkelfkde vrkiglelgl
      guaA_syn  ktgervavki kshhnvgglp knlrf..klv eplrklfkde vrklgrsigl
      guaA_h.p  ...kgpskvi kthhnvgglp ewmdf..kli eplrelfkde vrllgkelgv
    guaA_human  .vasgkaeli kthhndteli rklreegkvi eplkdfhkde vrilgrelgl
                451                                                  500
      guaA_N.t  SEDFLKRHPF PGPGLAVRIP GDVTAGNSLD ILRQVDEIFI QSIRDAKIYD
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
      guaA_e.c  pydmlyrhpf pgpglgvrvl gevkk.eycd llrradaifi eelrkadlyd
      guaA_syn  peeivrrhpf pgpglairii gevts.erln ilrdadfivr deiskrgiyh
      guaA_h.p  sqdflmrhpf pgpglavril geise.skik rlqeadfifi eelkkanlyd
    guaA_human  peelvsrhpf pgpglairvi c.aeepyick dfpetnnilk ivadfsasvk
                501                                                  550
      guaA_N.t  EIWQAFAVFL PVKTVGVQGD QRTHSHAVAL RA.VTSQDGM TADWYYFDFK
  guaA_est_A.t  ---------- ------aqgd kgtiphvgcp pcrlqaqvgl tadwfifehk
      guaA_e.c  kvsqaftvfl pvrsvgvmgd grkydwvvsl ra.vetidfm tahwahlpyd
      guaA_syn  dywqafavll pirsvgvmgd krtyahpvvl rf.itsedgm tadwarvpyd
      guaA_h.p  kvwqafcvll nvnsvgvmgd nrtyenaicl ra.vnasdgm tasfsflehs
    guaA_human  kphtllqrvk actteedqek lmqitslhsl nafllpiktv gvqgdcrsys
                551                                                  600
      guaA_N.t  FLDDVSRKIC NSVRGVNRVL LDITSKPPST IEWE------ ----------
  guaA_est_A.t  flddvsrkic nsvqgvnrvv lditskppst iewe------ ----------
      guaA_e.c  flgrvsnrii nevngisrvv ydisgkppat iewe------ ----------
      guaA_syn  ileaisnriv nevkgvnrvv yditskppgt iewe------ ----------
      guaA_h.p  flekvsnrit nevsginrvv yditskppgt iewe------ ----------
    guaA_human  yvcgisskde pdweslifla rliprmchnv nrvvyifgpp vkepptdvtp
                601                                                  650
      guaA_N.t  ---------- ---------- ---------- ---------- ----------
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
      guaA_e.c  ---------- ---------- ---------- ---------- ----------
      guaA_syn  ---------- ---------- ---------- ---------- ----------
      guaA_h.p  ---------- ---------- ---------- ---------- ----------
    guaA_human  tflttgvlst lrqadfeahn ilresgyagk isqmpviltp lhfdrdplqk
                651                                                  700
      guaA_N.t  ---------- ---------- ---------- ---------- ----------
  guaA_est_A.t  ---------- ---------- ---------- ---------- ----------
      guaA_e.c  ---------- ---------- ---------- ---------- ----------
      guaA_syn  ---------- ---------- ---------- ---------- ----------
      guaA_h.p  ---------- ---------- ---------- ---------- ----------
    guaA_human  qpscqrsvvi rtfitsdfmt gipatpgnei pvevvlkmvt eikkipgisr
                701       716
      guaA_N.t  ---------- ------
  guaA_est_A.t  ---------- ------
      guaA_e.c  ---------- ------
```

```
    guaA_syn  ---------- ------
    guaA_h.p  ---------- ------
  guaA_human  imydltskpp gttew
Exampl  2
```

Isolation of a cDNA of the guaA gene, coding for a GMP synthetase from the moss Physcomitrella patens

Double-stranded cDNA was generated from mRNA from protonemata of various ages of Physcomitrella patens and used to produce a cDNA bank in the vector pBluescript SKII (lambda ZAP II RI Library construction kit, Stratagene). Single clones from this bank were partially sequenced. The sequence of the clone 093-d11 showed clear homology with the GMP synthetase from Aquifex aeolicus. The complete sequence of 093-d11 was determined, see SEQ-ID No. 3. 093_d11 has a length of 1232 nucleotides and codes on a continuous reading frame for 382 amino acids. Comparison with GMP-7M reveals that 093_d11 is a partial cDNA. The homology with GMP-7M is 66.7% at the nucleotide level and 74.6% at the amino acid level.

Example 3

Demonstration of the function of GMP-7M by complementation of E. coli

The GMP-7M cDNA was employed as template for a PCR with the oligonucleotides 5'-CCTAGCCATGGAACCTCAAAC-3' and 5'-TATAGGATCCTACTTTGGTCACC-3'. The reaction mixtures contained about 0.1 ng of GMP-7M DNA, 0.5 µM of the appropriate oligonucleotides, 200 µM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM $MgCl_2$) and 0.02 U/µl Pfu polymerase (Stratagene).

The amplification conditions were set as follow:

Annealing temperature:	50°C, 30 sec
Denaturation temperature:	92°C, 30 sec
Elongation temperature:	72°C, 3 min
Number of cycles:	25

The resulting fragment of about 1670 bp was ligated via the NcoI and BamHI cleavage sites introduced by the oligonucleotides into the vector pTrc99A (Pharmacia). The resulting construct GMP-7Trc was transformed into the E.coli strain AT2465 (genetic markers: thi-1, guaA21, r lA1, λ, spoT1) and plated out on M9 minimal medium (Sambrook t al. (1989) Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) with and without 100 µg/ml guanosine.

The minimal media contained 0.4% glucose, 0.2% casamino acids, 100 µg/ml thiamine, 100 µg/ml inosine, 100 µg/ml biotin, 100 µg/ml histidine, 100 µg/ml arginine, 100 µg/ml 2'-deoxyuridine, 100 µM IPTG and 25 µg/ml ampicillin. The cloning vectore pTrc99A was transformed into AT2465 in a parallel experiment. It emerged that only the transformed bacteria which contained a GMP-7M cDNA from tobacco in the expression vector pTrc99A were capable of growth on minimal media without guanosine (see Tab. 2), which points strongly to the GMP-7M cDNA coding for an active GMP synthetase. The enzyme encoded by GMP-7M thus represents the first functional GMP synthetase isolated from plants.

Table 2

Growth of E. coli AT2465 transformed with various plasmids after 2 days at 37°C

	pTrc99A + GMP-7M	pTrc99A
Minimal medium without guanosine	+	-
Minimal medium with guanosine (100 µg/ml)	+	+

Example 4

Overexpression of the GMP synthetase from tobacco in E.coli and production of antibodies

For overexpression in E.coli, BamHI cleavage sites were introduced by PCR with GMP-7M as template and the oligonucleotides GMPA: 5'-GCAATGGATCCTCAAACACAGGCG-3' and GMPB: 5'-AAAAGGATCCTACTTTGGTCACC-3' and made it possible to clone the fragment in the vector pET15b (Novagen). A GMP-7M reading frame with hexahistidine anchor at the N terminus was produced in this way. After the correct orientation had been checked by restriction digestion and polymerase errors had been excluded by sequencing, the resulting construct GMP-7E was transformed into E. coli BL21(DE3) (Stratagene). IPTG-induced one-day cultures were harvested by centrifugation, and the cell pellets were lyzed and treated further in accordance with the manufacturer's information for nickel affinity chromatography ("Qia-Express-Kit", Qiagen). It was possible in this way to purify the GMP synthetase to more than 95% purity. The protein was used for producing antisera in rabbits by conventional

protocols (carried out on contract by Eurogent c, Herstal, Belgium).

Example 5

Expression of the GMP synthetase from tobacco in baculovirus-infected insect cells

In order to obtain sufficient active GMP synthetase for mass testing of chemicals, a 1.65 kb fragment was excized from GMP-7E with BamHI and cloned into the transfer vector pFastBacHTa (GibcoBRL). The resulting construct GMP-7I was used to generate recombinant baculovirus in accordance with the manufacturer's information (GibcoBRL). This virus was used in accordance with the manufacturer's information (GibcoBRL) for infecting Sf21 insect cells in order to produce active GMP synthetase whose activity could be measured after disruption of the cells in 50 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM EDTA, 10 mM PMSF and desalting of the extract on a Sephadex G-25 column (Pharmacia, Sweden).

Example 6

Production of plant expression cassettes

The antisense and cosuppression techniques were used with the aim of reducing the GMP synthetase activity in transgenic tobacco plants. For this purpose, plasmid constructs were produced in the vector pBinAR (Höfgen and Willmitzer, Plant Science (1990) 66, 221-230). A fragment of 1599 bp obtained from GMP-7M with *Bam*HI and *Bgl*II was ligated into the *Bam*HI-cut vector pBinAR. The 1599 bp fragment encodes the 5'-terminal part of the GMP synthetase cDNA. Clones obtained after transformation into E.coli XL1-blue were examined for the orientation of the 1599 cassette by cutting with *Hind*III as a check. The plasmids pGMP7AS (antisense construct) and pGMP7EX (sense construct) were identified in this way, see Figure 2.

Example 7

Generation and analysis of transgenic plants

The plasmids pGMP7AS and pGMP7EX - see Figure 2 - were transformed into Agrobacterium tumefaciens C58C1:pGV2260 (Deblaere et al., Nucl. Acids. Res. 13(1984), 4777-4788). A 1:50 dilution of an overnight cultur of a positively transformed agrobacterial colony in Murashige-Skoog medium (Physiol. Plant. 15(1962), 473) with 2% sucrose (2MS medium) was used for

transforming tobacco plants (Nicotiana tabacum cv. Samsun NN). Leaf disks from sterile plants (each about 1 cm^2) were incubat d in a Petri dish with a 1:50 agrobacteria dilution for 5-10 minutes. This was followed by incubation on 2MS medium with 0.8% Bacto agar in the dark at 25°C for 2 days. Cultivation was continued after 2 days with 16 hours light/8 hours dark, and continued in a weekly rhythm on MS medium with 500 mg/l Claforan (cefotaxime sodium), 50 mg/l kanamycin, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid and 1.6 g/l glucose. Growing shoots were transferred to MS medium with 2% sucrose, 250 mg/l Claforan and 0.8% Bacto agar. Regenerated shoots were obtained on 2MS medium with kanamycin and Claforan and, after rooting, were transferred into soil and, after cultivation for two weeks in a controlled-environment cabinet with a 16 hour light/8 hour dark rhythm at 60% humidity, investigated for foreign gene expression and altered metabolite contents and phenotypical growth traits. Altered nucleotide contents can be determined, for example, by the method of Stitt et al. (FEBS Letters, 145 (1982), 217-222).

The transgenic GMP synthetase antisense plants and their filial generation showed reduced growth, compared with WT plant controls, and bleaching of the sink leaves. These phenotypical changes occurred in an early growth stage (see Fig. 3). In plants with reduced growth it was possible to detect in Northern hybridization a reduced amount of GMP-7M RNA, compared with the wild type. 40 µg portions of complete RNA from sink leaves were employed for this purpose. Complete RNA was isolated from plant tissues as described by Logemann et al. (Anal. Biochem. 163 (1987), 21). For the analysis in each case 40 µg of RNA were fractionated in a formaldehyde-containing 1.5% agarose gel and transferred to nylon membranes (Hybond, Amersham). Specific transcripts were detected as described by Amasino (Anal. Biochem. 152(1986), 304). A specific c-DNA probe of the antisense strand was generated. This was done by cleaving the plasmid GMP-7M with BamHI and BglII and isolating a fragment comprising 1600 bp. The oligonucleotide 5'-GAT ACG TCG TCA AGG AAC TTG-3' was used for the labeling reaction. The probe was hybridized by standard methods, see Hybond information for users, Amersham. Hydridization [sic] signals were visualized by autoradiography using Kodak X-OMAT AR films. A clear correlation between expression of the growth phenotype and a reduction in the amount of GMP-7M RNA was shown (Fig. 3).

It was moreover possible in a Western blot experiment to detect a reduced amount, compared with wild-type plants, of GMP synthetase in the transgenic lines. This was done by preparing total protein extracts from sink leaves, separating in SDS polyacrylamide g l

electrophoresis by standard methods and transferring to nitrocellulose membranes. Detection took place with an IgG-alkaline phosphatase conjugat and the BCIP/NBT system (Sigma).

In addition, it was possible by the in vitro assay described in Example 8 to establish that there was reduced GMP synthetase activity in transgenic lines with reduced growth.

The correlation between the level of expression and the GMP synthetase activity and growth phenotype suggest that GMP synthetase is a suitable target for herbicides.

Example 8

Assay systems for measuring GMP synthetase activity

The systems developed by Spector (Methods in Enzymology LI, 1978, 219-224) for animal enzymes can be used to measure plant GMP synthetase activity. In the first system, the AMP formation is made possible by coupling the reaction with AMP kinase, pyruvate kinase, lactate dehydrogenase and measurement at 340 nm. The second system is based on direct detection of GMP (guanosine monophosphate) by employing the radiolabeled substrate XMP (xanthine monophosphate) and fractionation by thin-layer chromatography.

Alternatively, the GMP synthetase activity can also be measured by a novel system, namely coupled detection of the produced glutamate. This system has the advantage of a smaller number of coupled reaction steps and provides greater signal strengths.

$$XMP + ATP + L\text{-glutamine} + H_2O \xrightarrow{GMP\text{-}S} GMP + AMP + L\text{-glutamate} + PP_i$$

$$L\text{-glutamate} + APAD + H_2O \xrightarrow{GluDH} oxoglutarate + APADH + NH_4^+$$

(GMP-S = GMP synthetase, GluDH = glutamate dehydrogenase, APAD = 3-acetylpyridine adenine dinucleotide)

For this, the reaction mixture (see below) was incubated at 37°C for 60 minutes, and the reaction was stopped by incubation at 95°C for 5 minutes. The glutamate formed was d tected in the detection mixture (see below) by photometric measurement of the increase in APADH at 363 nm.

Reaction mixture:

100 µL	750 mM	Tris/HCl buffer pH 7.8
100 µL	100 mM	$MgCl_2$
100 µL	80 mM	KCl
100 µL	20 mM	XMP
100 µL	200 mM	L-glutamine
400 µL		H_2O
100 µL		protein extract
1000 µL		

Detection mixture:

375 µL	100 mM	Tris-HCl buffer pH 8.0
75 µL	500 mM	KCl
125 µL		H_2O
75 µL	3 mM	APAD
100 µL		of the reaction mixture
750 µL		

Example 9

Search for inhibitors of GMP synthetase activity

The *in vitro* assay described in Example 8 can be used with high throughput methods to search for inhibitors of GMP synthetase activity. The GMP synthetase activity for this can be prepared from plant tissues. It is possible and preferred for a plant GMP synthetase to be expressed in E.coli, insect cells or another suitable expression system and then be concentrated or isolated. It was possible in this way to identify known inhibitors such as 6-thio-XMP.